

**AD-A235 474****TATION PAGE**Form Approved  
OMB No 0704-0188

to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering the collection of information. Send comments regarding this burden estimate or any other aspect of this form, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Avenue, Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

DATE  
103. REPORT TYPE AND DATES COVERED  
Reprint

## 4. TITLE AND SUBTITLE

(see title on reprint)

## 5. FUNDING NUMBERS

Program Element No.  
NWED QAXM

## 6. AUTHOR(S)

McKinney, L.C., and Gallin, E.K.

Work Unit No.  
00020

## 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Armed Forces Radiobiology Research Institute  
Defense Nuclear Agency  
Bethesda, MD 20889-51458. PERFORMING ORGANIZATION  
REPORT NUMBER

SR91-18

## 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Defense Nuclear Agency  
Washington, DC 2030510. SPONSORING/MONITORING  
AGENCY REPORT NUMBER

## 11. SUPPLEMENTARY NOTES

## 12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

## 12b. DISTRIBUTION CODE

## 13. ABSTRACT (Maximum 200 words)



ABSTRACT FOR

NTIS GRA&amp;I

DTIC TAB

Unannounced

Justification

By

Distribution

Availability Code

Distribution

List of Special

A-1 20

## 14. SUBJECT TERMS

## 15. NUMBER OF PAGES

10

## 16. PRICE CODE

17. SECURITY CLASSIFICATION  
OF REPORT

UNCLASSIFIED

18. SECURITY CLASSIFICATION  
OF THIS PAGE

UNCLASSIFIED

19. SECURITY CLASSIFICATION  
OF ABSTRACT20. LIMITATION OF  
ABSTRACT

## Effect of Adherence, Cell Morphology, and Lipopolysaccharide on Potassium Conductance and Passive Membrane Properties of Murine Macrophage J774.1 Cells

Leslie C. McKinney and Elaine K. Gallin

Department of Physiology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814

**Summary.** The effects of adherence, cell morphology, and lipopolysaccharide on electrical membrane properties and on the expression of the inwardly rectifying K conductance in J774.1 cells were investigated. Whole-cell inwardly rectifying K currents ( $I_K$ ), membrane capacitance ( $C_m$ ), and membrane potential ( $V_m$ ) were measured using the patch-clamp technique. Specific  $K_i$  conductance ( $G_K$ , whole-cell  $K_i$  conductance corrected for leak and normalized to membrane capacitance) was measured as a function of time after adherence, and was found to increase almost twofold one day after plating. Membrane potential ( $V_m$ ) also increased from  $-42 \pm 4$  mV ( $n = 32$ ) to  $-58 \pm 2$  mV ( $n = 47$ ) over the same time period.  $G_K$  and  $V_m$  were correlated with each other;  $G_L$  (leak conductance normalized to membrane capacitance) and  $V_m$  were not. The magnitudes of  $G_K$  and  $V_m$  15 min to 2 hr after adherence were unaffected by the presence of 100  $\mu$ M cycloheximide, but the increase in  $G_K$  and  $V_m$  that normally occurred between 2 and 8 hr after adherence was abolished by cycloheximide treatment. Membrane properties were analyzed as a function of cell morphology, by dividing cells into three categories ranging from small round cells to large, extremely spread cells. The capacitance of spread cells increased more than twofold within one day after adherence, which indicates that spread cells inserted new membrane. Spread cells had more negative resting membrane potentials than round cells, but  $G_K$  and  $G_L$  were not significantly different. Lipopolysaccharide (LPS; 1 or 10  $\mu$ g/ml) treated cells showed increased  $C_m$  compared to control cells plated for comparable times. In contrast to the effect of adherence, LPS-treated cells exhibited a significantly lower  $G_K$  than control cells, indicating that the additional membrane did not have as high a density of functional  $G_K$  channels. We conclude that both adherence and LPS treatment increase the total surface membrane area of J774 cells and change the density of  $K_i$  channels. In addition, this study demonstrates that membrane area and density of  $K_i$  channels can vary independently of one another.

**Key Words** potassium channel · macrophage · J774.1 cells · lipopolysaccharide · adherence · ion transport

### Introduction

A number of different K currents have been described in macrophages or macrophage-like cell lines (for review see Gallin & McKinney, 1989).

Several of these studies have shown that ionic currents change with the functional state of the cell. In human peripheral blood monocytes, large conductance Ca-activated K channels appear in the plasma membrane over a time course of 4–5 days as the cells mature from monocytes into macrophages (Gallin & McKinney, 1988), while different, smaller conductance Ca-activated K channels are present both shortly after isolation and after 4–5 days in culture (Gallin, 1989). In addition, exposure of human monocytes to lipopolysaccharide (LPS), an 'activating' or 'priming' stimulus, increases the percentage of cells expressing a transient outward K current ( $K_o$ ) from 0 to approximately 30% (Jow & Nelson, 1989). In the murine macrophage-like cell line J774.1, a  $K_o$  current is briefly expressed immediately after cells become adherent, while an inwardly rectifying K current ( $K_i$ ) increases in magnitude over a time course of approximately one day (Gallin & Sheehy, 1985). Adherent mouse peritoneal macrophages also express a  $K_i$  current after five days in culture (Randriamampita & Trautmann, 1987), but do not appear to express this conductance before that time (Ypey & Clapham, 1984).

These studies demonstrate that the state of maturation of the macrophage, as well as adherent culture conditions or exposure to LPS, can affect the expression of K channels in macrophages. It is well known that adherence induces functional changes in macrophages that are associated with cell activation. (For a general discussion of macrophage activation see Cohn (1978) or Karnovsky and Lazdins (1978).) These include increased synthesis or secretion of various lymphokines (Fuhlbrigge et al., 1987; Haskill et al., 1988), and an increase in the oxidative burst induced by phorbol myristate acetate (PMA; Berton & Gordon, 1983; Kunkel & Duque, 1983). Other adherence-induced changes include an increase in resting membrane potential (Sung et al., 1985), increased amino acid transport

(Pofit & Strauss, 1977), transiently increased levels of IP<sub>3</sub> (Zabrenetzky & Gallin, 1988), development of peroxidase activity within 2 hr post-adherence (Bodel, Nichols, & Bainton, 1977), and expression of several surface antigens that are not expressed in cells in suspension (Triglia, Burns & Werkmeister, 1985; Midoux et al., 1989).

Exposure to LPS also induces a variety of changes in macrophages and macrophage cell lines, including increased spreading (Pabst & Johnston, 1980) and membrane ruffling of adherent cells (Morland & Kaplan, 1977), and stimulation of lymphokine synthesis and secretion (Zacharchuk et al., 1983; Fuhlbrigge et al., 1987). Most importantly, LPS 'primes' the cell to respond to other stimuli (Gordon, Unkeless & Cohn, 1974; Pabst, Hede-gard & Johnston, 1982). In J774.1 cells, LPS inhibits cell division (Ralph & Nakoinz, 1977; Kurland & Bockman, 1978; Okada et al., 1978) and induces secretion of prostaglandin E (Kurland & Bockman, 1978) and T-cell activating factors (Okada et al., 1978).

The purpose of this study was to determine the effects of both adherence and LPS on the expression of the inwardly rectifying K conductance and on the electrical membrane properties (membrane potential,  $V_m$ ; leak resistance,  $R_L$ ; and capacitance,  $C_m$ ) of the murine macrophage-like cell line J774.1. While Gallin and Sheehy (1985) noted that the magnitude of  $K_i$  in J774.1 cells increased with time after adherence, they did not determine whether the increased current magnitude was due to an increase in cell size, or whether current density (current/unit membrane area) increased. This question was resolved in the present study by directly measuring whole-cell inwardly rectifying K conductance and normalizing this value to  $C_m$  to obtain specific  $K_i$  conductance ( $G_K$ ). Since membrane capacitance is directly proportional to membrane area, these measurements also yielded information about the effect of adherence and LPS on the insertion of new membrane by the cell. We also examined the effect of cycloheximide, a protein synthesis inhibitor, on the expression of  $G_K$  following adherence, since protein synthesis inhibitors have been shown to interfere with the expression of other surface antigens in the macrophage (Smith & Ault, 1981; Triglia et al., 1985). Finally, the expression of  $G_K$  as a function of cell shape was characterized, to determine if  $G_K$  was differentially expressed in very spread *versus* nonspread cells.

## Materials and Methods

### CELL CULTURE

J774.1 (J774A.1) cells were obtained from American Type Tissue Culture (Rockville, MD) and maintained in suspension at 37°C

for not more than 40 days. Cells were fed at least 12 hr prior to plating for electrophysiological experiments. J774.1 cells have a doubling time of <24 hr and were not synchronized with respect to cell cycle. Whittaker RPMI 1640 culture medium (Bioproducts, Walkersville, MD) was supplemented with 5% fetal calf serum, 4 mM glutamine, and 100 U/ml penicillin-streptomycin (DIFCO). For recording, cells were placed on glass coverslips and maintained in culture medium for varying times (minutes to days). Prior to patch-clamp recordings, coverslips were mounted in a plexiglass chamber in 300–400  $\mu$ l of Na Hanks', maintained at room temperature (23 to 26°C), and the recording media changed every 20 to 30 min. Coverslips were recorded from for no more than 1 hr.

### RECORDING METHODS

Recording methods were the same as those previously described (McKinney & Gallin, 1988). Briefly, whole-cell current records were obtained using a List (Darmstadt, FRG) EPC-7 patch clamp. Voltage pulses were generated by computer and currents were digitized, displayed, and analyzed using an Indec Laboratory Display System (Sunnyvale, CA). Patch electrodes of 3–5 M $\Omega$  resistance were made from hematocrit glass (#02-668-68, Fisher Scientific, Pittsburgh, PA). Zero current potentials were measured in current clamp mode immediately after attainment of the whole-cell configuration, and cells were held within 5 mV of this value.

### ANALYTICAL METHODS

Whole-cell records were corrected for leak and capacity currents. Electrode capacitance was compensated while in the cell-attached mode. Total membrane capacitance ( $C_m$ ) was measured in the whole-cell mode by integrating the capacity transient and was then compensated electronically. Series resistance ( $R_s$ ) was measured either directly from the EPC-7 after capacity transient cancellation or by fitting a capacitance record with a mono-exponential function to find  $\tau$  and calculating  $R_s$  from the relationship  $R_s = \tau/C$ . The average series resistance for 95 cells was  $6.9 \pm 0.6$  M $\Omega$ . Leak current was measured in one of two ways: by measuring the current in the voltage range –45 to –32 mV where no time-dependent current was present, or by measuring the current at negative voltages in the presence of 2 mM barium, which blocks all inwardly rectifying current and results in a linear  $I$ – $V$  relationship. Leak resistances ( $R_L$ ) obtained using either method were not significantly different from one another:  $R_L = 2.7 \pm 0.3$  G $\Omega$  ( $n = 89$ ) in the absence of barium and  $R_L = 3.0 \pm 0.3$  G $\Omega$  ( $n = 39$ ) in the presence of barium. For purposes of comparison to inwardly rectifying K conductance measurements, leak resistance values were converted to units of conductance [ $R_L$  (G $\Omega$ ) =  $1/G$  (nS)]. Specific leak conductance ( $G_L$ ) was determined by dividing the leak conductance by total membrane capacitance. As in our previous study (McKinney & Gallin, 1988), for calculations of channel density (# channels/ $\mu$ m<sup>2</sup> of membrane), the specific capacitance of the cell membrane was assumed to be 1  $\mu$ F/cm<sup>2</sup>. Values of surface area calculated using this value compare well with values reported for mouse peritoneal macrophages obtained by stereologic analysis (Steinman, Brodie & Cohn, 1976). Values of surface area obtained using either capacitance measurements or stereologic techniques are two- to three-fold larger than surface area measurements calculated from values of cell diameter (assuming a spherical cell shape). This is because macrophages are irregularly shaped and can have considerable membrane ruffling and infolding.

Whole-cell conductance for inward current ( $K_i$ ) was measured in the following way. Peak current amplitude was measured at the beginning of a hyperpolarizing voltage step by eye using a cursor. Current vs. voltage ( $I$ - $V$ ) curves were constructed and a straight line fitted to the data for steps between  $-90$  to  $-160$  mV to yield a conductance value. After subtraction of leak conductance, values were normalized to membrane capacitance to yield a value for specific  $K_i$  conductance  $G_{K_i}$ .

In order to get an accurate sampling of whole-cell conductance values from the population of J774.1 cells, cells were not excluded on the basis of low resting membrane potentials or small inward current amplitudes. After verifying increased capacitance after rupture of the patch (the lowest value accepted was 11 pF) and an acceptable series resistance value (not greater than 20 M $\Omega$ ) for a particular recording, we included virtually all cells that had stable membrane properties in the study.

### $^3\text{H}$ -LEUCINE INCORPORATION

The effect of cycloheximide on protein synthesis was assayed by measuring incorporation of  $^3\text{H}$ -leucine into protein in the presence of varying concentrations of the protein synthesis inhibitor cycloheximide (Sigma Chemical, St. Louis, MO). An  $\text{IC}_{50}$  of approximately 1  $\mu\text{M}$  was observed; 100  $\mu\text{M}$  cycloheximide inhibited greater than 90% of  $^3\text{H}$ -leucine incorporation in these cells.

### SOLUTIONS

Cells were bathed in a Na Hanks' solution consisting of (in mM): 145 NaCl, 4.5 KCl, 1.6  $\text{CaCl}_2$ , 1.1  $\text{MgCl}_2$ , 10 HEPES NaOH buffer, pH 7.3. The pipette contained (in mM): 145 KCl, 1  $\text{MgCl}_2$ , 1.1 EGTA, 0.1  $\text{CaCl}_2$ , and 10 HEPES KOH, pH 7.3. Free Ca in this solution was less than  $10^{-8}$  M. *Escherichia coli* derived lipopolysaccharide (LPS) was obtained from DIFCO Laboratories (Detroit, MI).

### STATISTICAL METHODS

Unless otherwise stated, mean values were considered to be significantly different from one another if  $P < 0.05$  using a Student's  $t$  test. Linear regression analysis was carried out on a VAX 11/750 using the RSI statistical package (BBN Software Products, Cambridge, MA). Two values were said to be correlated if the  $R$  value of the slope of the line relating the two variables was significantly different from zero ( $P < 0.05$ ). In some cases, in order to analyze upward or downward trends in the data with time, biweight regressions were done and slopes were compared to zero. Slopes of control vs. test data were compared to each other using a  $t$  test.

### Results

#### MEMBRANE CAPACITANCE, SPECIFIC $K_i$ CONDUCTANCE, AND MEMBRANE POTENTIAL INCREASE WITH TIME AFTER ADHERENCE

Figure 1A shows an example of inwardly rectifying  $K_i$  currents recorded from J774.1 cells bathed in Na Hanks, with the corresponding  $I$ - $V$  relationship shown in Fig. 1B. The  $K_i$  current activates at  $-50$

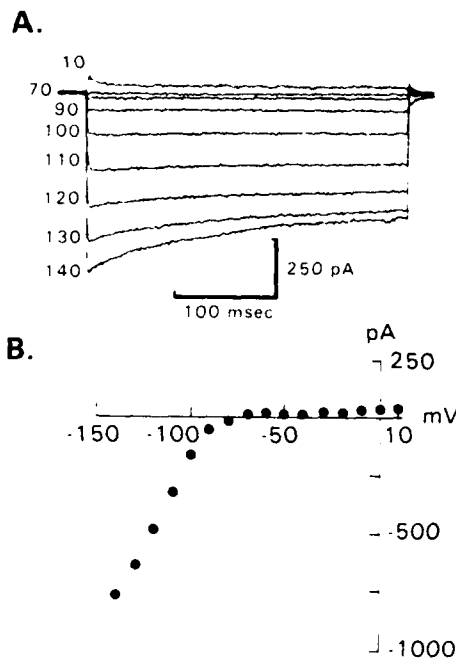
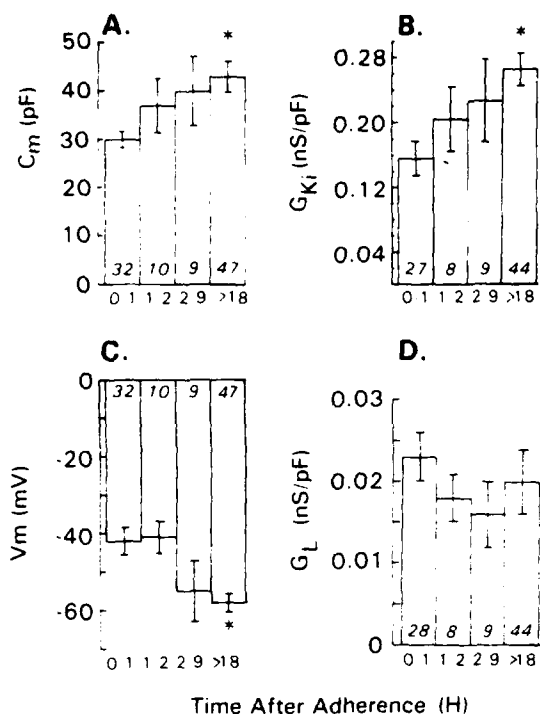


Fig. 1. (A) Inwardly rectifying  $K_i$  current. Cell was held at  $-70$  mV and 440 msec voltage pulses applied in 10-mV increments. Records have not been leak subtracted to show total current. (B) Peak current-voltage plot for experiment shown in A.

mV, and shows time-dependent inactivation below about  $-120$  mV that is partially due to block by Na $^+$  and partially due to inactivation of the current (McKinney & Gallin, 1988). Properties of both the whole-cell current and the single-channel events underlying them have been described in detail elsewhere (McKinney & Gallin, 1988). The  $K_i$  current is completely blocked by 1 mM barium and in >95% of the recordings was the only time-dependent current observable under these recording conditions. Therefore, the leak-subtracted whole-cell conductance measured over negative voltages appears to be due solely to the conductance of the  $K_i$  current. An inactivating outward  $K$  current ( $K_o$ ) was observed in about 5% of cells, but it activated at potentials above  $-40$  mV (Gallin & Sheehy, 1985), and did not interfere with measurements of the inwardly rectifying current. Randriamampita and Trautmann (1987) have reported the existence of a linear Ca-dependent  $K$  current in J774 cells which is apparent only when intracellular calcium is buffered at 1  $\mu\text{M}$ . In our experiments,  $[\text{Ca}]_i$  was buffered at  $10^{-8}$  M and so this current was not observed.

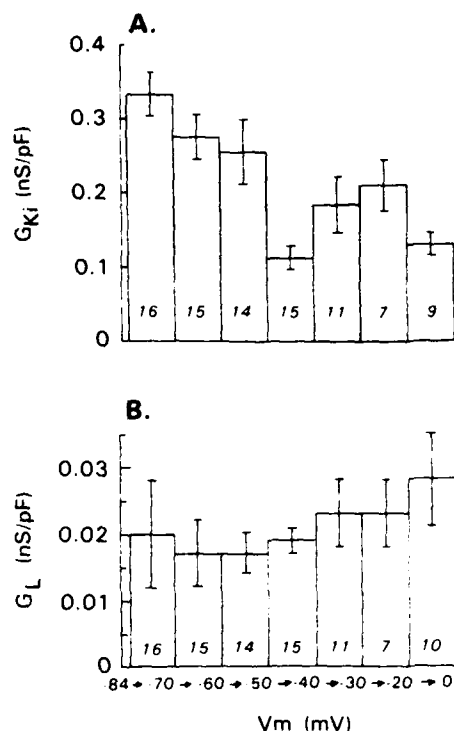
In order to study how the  $K_i$  conductance and other membrane properties change after adherence, whole-cell recordings were obtained at varying times after plating. Values for  $C_m$ ,  $G_{K_i}$ ,  $V_m$ , and  $G_{K_i}$  are plotted in the form of bar graphs showing the mean  $\pm$  SEM as a function of time after adherence



**Fig. 2.** Plots of (A)  $C_m$ , (B)  $G_{K_i}$ , (C)  $V_m$ , and (D)  $G_L$  vs. time after adherence. Values plotted are means  $\pm$  SEM. Asterisk (\*) indicates values which are significantly different from values at 0-1 hr. Numbers within the bar, for these and all subsequent graphs, indicate number of cells studied.

(Fig. 2). Cells were grouped into four time periods: 0-1 hr (to follow changes in membrane properties at early times after adherence), 1-2, 2-9, and >18 hr. It should be noted that the earliest time point at which recordings were made was approximately 15 min after adherence, and no recordings were made at times longer than about 36 hr after adherence.

Significant changes in membrane properties were noted following adherence. Membrane capacitance increased significantly with time after plating (Fig. 2A) from  $30 \pm 2$  pF to  $43 \pm 3$  pF. Assuming that  $1 \text{ cm}^2$  of membrane has a capacitance of  $1 \mu\text{F}$ , the average membrane area of the cells increased by about 40%. Thus, the morphological changes which J774.1 cells undergo after adherence do not merely represent shape changes but reflect a net insertion of additional membrane.  $G_{K_i}$  increased almost two-fold over 18 hr (Fig. 2B), from  $0.16 \pm 0.02$  nS/pF to  $0.27 \pm 0.02$  nS/pF. Since this value is normalized to membrane area, the increase in  $G_{K_i}$  was not due to the increased membrane area of the cells. Our single-channel data indicate that the conductance of single inwardly rectifying channels does not change after adherence (*data not shown*). Therefore, the increase in  $G_{K_i}$  represents an increase in the density of  $K_i$  channels in the membrane over this time period from 44 channels/pF ( $0.44 \text{ channels}/\mu\text{m}^2$ ) to 75



**Fig. 3.** (A) Plot of  $G_{K_i}$  vs. resting  $V_m$ . (B) Plot of  $G_L$  vs.  $V_m$ . Conductance values given are mean  $\pm$  SEM.  $V_m$  values (in mV) were grouped as follows: -84 to -70, -69 to -60, -59 to -50, -49 to -40, -39 to -30, -29 to -20, -19 to 0.

channels/pF ( $0.75 \text{ channels}/\mu\text{m}^2$ ). These data were supported by direct observations of  $K_i$  channel activity in cell-attached patches (McKinney & Gallin, 1988). Membrane patches from freshly plated cells usually contained few (0-3) channels, while membrane patches from cells adherent for one day usually contained many (2-6) channels. Since both channel density and membrane area increased with time after adherence, the average number of channels per cell increased from 1,320 to 3,225 over 18 hr.

During the 18-hr period following adherence, the average  $V_m$  of the cells increased from  $-42 \pm 4$  mV to  $-58 \pm 2$  mV (Fig. 2C). The increase in membrane potential could be due to an increase in  $G_{K_i}$  or could also be due to a concomitant decrease in  $G_L$ . However, as shown in Fig. 2D,  $G_L$  did not change significantly with time after adherence, indicating that the cells maintained a rather constant leak conductance over the time that  $C_m$ ,  $V_m$ , and  $G_{K_i}$  were increasing.

#### SPECIFIC $K_i$ CONDUCTANCE AND MEMBRANE POTENTIAL ARE CORRELATED

Figure 3 shows the relationship between  $G_{K_i}$  and resting  $V_m$ . (Note: resting  $V_m$  is actually determined

**Table 1.** Effect of cycloheximide on membrane properties of J774.1 cells

	Time after adherence (hr)	$G_K$ (nS/pF)	$G_L$ (nS/pF)	$V_m$ (mV)	$C_m$ (pF)
Control	0-2	$0.16 \pm 0.02$ (35)	$0.02 \pm 0.003$ (36)	$-42 \pm 3$ (42)	$31 \pm 2$ (42)
Cycloheximide-treated	0-2	$0.25 \pm 0.04$ (7)	$0.03 \pm 0.006$ (8)	$-51 \pm 6$ (10)	$35 \pm 4$ (10)
Control	2-9	$0.23 \pm 0.05$ (9)	$0.02 \pm 0.004$ (9)	$-55 \pm 8$ (9)	$40 \pm 7$ (9)
Cycloheximide-treated	2-8	$0.14 \pm 0.04$ (14)	$0.03 \pm 0.006$ (15)	$-54 \pm 5$ (15)	$30 \pm 2$ (15)

Note that cells were exposed to cycloheximide for 1 hr prior to plating, as well as during plating.

by steady state, not peak K conductance. However, over the voltage range of  $-50$  to  $-110$  mV, peak and steady-state conductance are equivalent.) Data are from cells plated at all times. Between  $-84$  and  $-40$  mV there was a positive correlation between  $G_K$  and resting  $V_m$ . That is, over the range at which the inwardly rectifying K conductance is activated, cells which had higher conductance to  $K_i$  also had a more negative resting membrane potential. In contrast, there was no correlation between  $G_K$  and  $V_m$  for voltages positive to  $-40$  mV. In addition, there was no correlation between  $V_m$  and  $G_L$  over any voltage range. This result indicates that cells which had low resting membrane potentials were not depolarized merely because they were 'leakier'. Likewise, there was no correlation between  $G_K$  and  $G_L$ ; cells with a low specific  $K_i$  conductance were not necessarily those with a large leak conductance. Thus, it is likely that the increase in resting  $V_m$  observed over the first 24 hr of adherence is a function of the increased conductance to  $K_i$ , and not a function of a change in  $G_L$ .

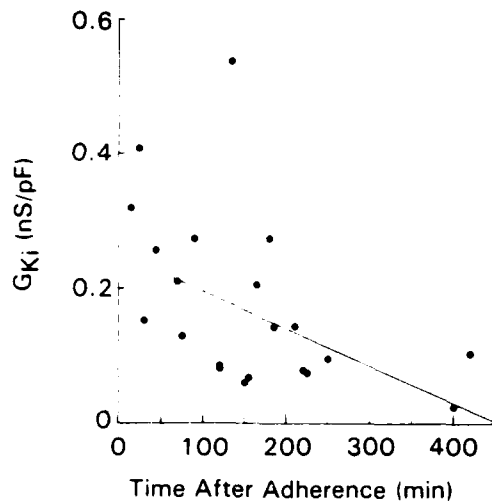
#### INITIAL EXPRESSION OF $G_K$ IS NOT INHIBITED BY CYCLOHEXIMIDE

To determine (i) whether the observed increase in the density of  $K_i$  channels after adherence depended on protein synthesis and (ii) whether inhibition of protein synthesis before cells were allowed to become adherent would interrupt the initial expression of  $K_i$ ,  $G_K$  was measured in cells treated with the protein synthesis inhibitor cycloheximide, which was shown to inhibit  $^3\text{H}$ -leucine incorporation in these cells (see Materials and Methods). Cells were exposed to cycloheximide ( $100 \mu\text{M}$ ) for 1 hr prior to plating, plated in the presence of cycloheximide, and  $G_K$  was measured at various times after plating. Cells treated with cycloheximide were able to adhere and spread similar to untreated cells, and the general morphology appeared normal. However, Gigaohm seals were considerably less stable, which made recording from the cells for an

extended period of time difficult. Values for  $G_K$ ,  $G_L$ ,  $V_m$  and  $C_m$  are given in Table 1, grouped into two time periods: 0-2 and 2-8 hr after adherence. Because prolonged incubation in cycloheximide may affect cell viability, the conductance of cells which had been plated in cycloheximide for more than 8 hr was not measured. Cycloheximide did not inhibit the initial expression of inward current. Average  $G_K$  0-2 hr after adherence was not significantly different from the average  $G_K$  value for untreated cells plated for the same amount of time. Values for  $G_L$ ,  $V_m$  and  $C_m$  were also not different from controls. However, with time after adherence, cycloheximide did inhibit the increase in  $G_K$  and  $V_m$  normally observed in control cells. While control cells increased their mean  $G_K$  from 0.16 to 0.23 nS/pF 2-9 hr after adherence, the mean  $G_K$  of cycloheximide-treated cells decreased from 0.25 to 0.14 nS/pF. When  $G_K$  values were plotted vs. time for cycloheximide-treated cells, the slope of the line was found to decline significantly (Fig. 4). Cycloheximide-treated cells did not show a significant trend to more negative resting membrane potentials with time after adherence, nor was there a significant trend toward increased membrane capacitance as was shown by control cells.  $G_L$  of cycloheximide-treated cells was unchanged after adherence. These data indicate that cycloheximide does not interfere with the initial expression of the inwardly rectifying K conductance, but does inhibit the upregulation of K channels and the insertion of new membrane which occurs following adherence.

#### DIFFERENT MORPHOLOGICAL GROUPS HAVE DIFFERENT MEMBRANE PROPERTIES

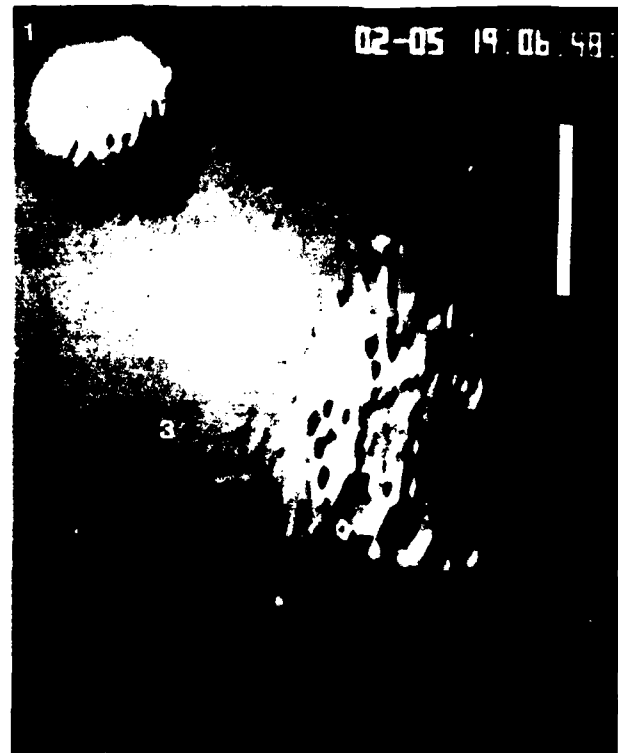
Adherent J774.1 cells exist in a variety of shapes and sizes that may reflect different functional states. For instance, cells undergoing cell division become rounded while migrating cells have a polarized appearance, with a leading and trailing edge. Other cells exhibit a very flattened appearance almost immediately after adherence. In order to de-



**Fig. 4.** Plot of  $G_{K_i}$  vs. time after adherence for cycloheximide-treated cells. Solid line is a linear biweight fit to the data. Its slope is significantly different from zero. Note that cells were exposed to cycloheximide 1 hr prior to plating, as well as during plating

termine whether cells of different morphologies had different membrane properties, cells were divided into three groups according to shape (see photograph, Fig. 5): group #1, small round cells without visible processes, mean capacitance  $27 \pm 3$  pF ( $n = 20$ ), average diameter  $22 \mu\text{m}$ ; group #2: somewhat spread or polarized cells, some with visible processes, mean capacitance  $36 \pm 2$  pF ( $n = 40$ ), average dimensions  $37 \times 28 \mu\text{m}$ ; and group #3: extremely spread cells, mean capacitance  $45 \pm 4$  pF ( $n = 36$ ), average dimensions  $50 \times 42 \mu\text{m}$ .  $V_m$ ,  $C_m$ ,  $G_K$ , and  $G_L$  for each morphological category were determined for two time periods: 0–2 and >18 hr after adherence (Fig. 6). Examining the data in this way revealed some interesting differences between cell types that were not apparent when the data from all cells were pooled (as in Fig. 2).

Values for  $V_m$ ,  $C_m$ ,  $G_K$ , and  $G_L$  were not significantly different between groups 1 and 2 at either 0–2 hr or >18 hr after adherence. Thus, for clarity, only data from groups 1 and 3 are shown in Fig. 6. Very spread cells were different from round cells in several respects. They were significantly more hyperpolarized than round cells at either 0–2 or >18 hr following adherence (Fig. 6A). Also, one day after adherence, spread cells had much larger membrane capacitance than round cells (Fig. 6B). Both round and spread cells still showed a positive correlation between  $V_m$  and  $G_K$ . However,  $G_K$  (Fig. 6C) and  $G_L$  (Fig. 6D) were not significantly different between groups 1 and 3 compared at the same time period (0–2 or >18 hr).



**Fig. 5.** Photograph of adherent J774.1 cells showing different morphological categories. Bar:  $20 \mu\text{m}$

We also examined how membrane properties changed with time after adherence for each group. First, only the very spread cells showed a significant (almost twofold) increase in membrane capacitance over 18 hr (Fig. 6B). Thus, most of the previously observed increase in membrane area which occurs after adherence (see Fig. 2A) can be attributed to the increased membrane area of very spread cells. Second, membrane potential significantly increased with time after adherence for both groups of cells (Fig. 6A). Round cells showed the largest increase in  $V_m$ , and also showed the largest increase in  $G_K$  18 hr after adherence (Fig. 6C). Spread cells showed a smaller increase in  $V_m$ , and a correspondingly smaller increase in  $G_K$ .  $G_L$  values did not change after adherence for either round or spread cells (Fig. 6D). We conclude that very spread cells behave differently from round cells after adherence: they insert more membrane but do not significantly increase  $K_i$  current density after adherence.

#### LPS-TREATED CELLS HAVE DIFFERENT MEMBRANE PROPERTIES

The effect of LPS on membrane properties of J774.1 cells was examined by incubating cells in suspen-

**Table 2.** Effect of LPS on membrane properties of J774.1 cells

	Time after adherence (hr)	$G_K$ (nS/pF)	$G_L$ (nS/pF)	$V_m$ (mV)	$C_m$ (pF)
Control	0-2	$0.16 \pm 0.02$ (35)	$0.02 \pm 0.003$ (36)	$-42 \pm 3$ (42)	$31 \pm 2$ (42)
LPS-treated	0-2	$0.09 \pm 0.02$ (13)	$0.02 \pm 0.007$ (14)	$-37 \pm 5$ (16)	$42 \pm 5$ (17)
Control	>18	$0.27 \pm 0.02$ (44)	$0.02 \pm 0.004$ (44)	$-58 \pm 2$ (47)	$43 \pm 3$ (47)
LPS-treated	>18	$0.15 \pm 0.02$ (8)	$0.02 \pm 0.006$ (9)	$-62 \pm 4$ (10)	$68 \pm 10$ (10)

<sup>a</sup> Significantly different from control cells at the same time period.

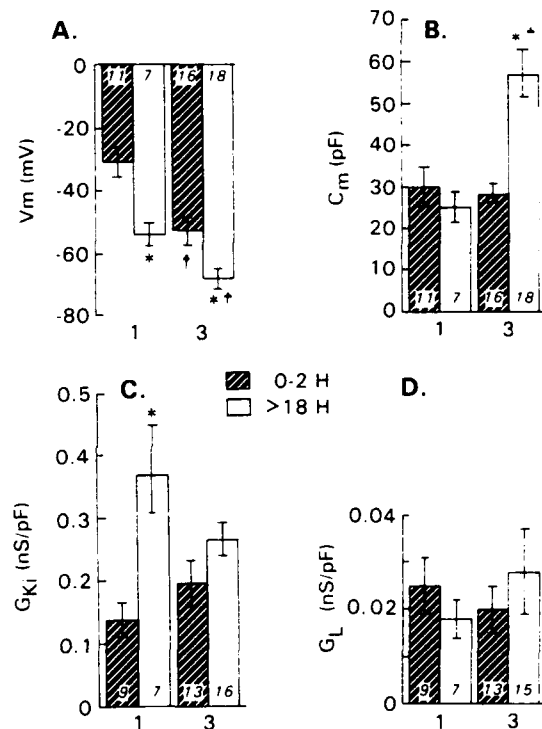
<sup>b</sup> Significantly different from the value at 0-2 hr.

Note that cells were exposed to LPS one day prior to plating, as well as during plating.

sion with 1 or 10  $\mu\text{g/ml}$  of LPS overnight and then plating cells for various times in the presence of LPS. Values for  $G_K$ ,  $G_L$ ,  $V_m$  and capacitance are given in Table 2, grouped into two time periods, 0-2 and 18 hr after adherence. Data were not separated into different morphological categories; LPS-treated cells were of all three groups but primarily of group #2. For a given time period after adherence,  $V_m$  and  $G_L$  values were comparable to those of control cells, but  $G_K$  values were significantly lower. However,  $G_K$  and  $V_m$  were still correlated;  $G_L$  and  $V_m$  were not. Membrane capacitance was larger, indicating that LPS-treated cells have a larger membrane surface area. Thus, activation of cells by LPS resulted in the insertion of new membrane, but did not result in increased density of K channels. Membrane properties of LPS-treated cells showed changes with time after adherence in a manner similar to control cells.  $G_K$ ,  $V_m$  and  $C_m$  all increased significantly one day after adherence, as they did for untreated cells, while  $G_L$  was unchanged.

## Discussion

This study demonstrates that membrane properties of J774.1 cells vary with time after adherence, cell morphology, and after exposure to LPS. Values for membrane capacitance ( $C_m$ ), membrane potential ( $V_m$ ), and specific K conductance ( $G_K$ ) were significantly increased 18 hr after adherence. As shown in Fig. 1, these trends were evident at times earlier than 18 hr post-adherence, even though statistically significant differences between mean values were not yet demonstrable. As a point of comparison, previous studies in T cells demonstrated that stimulation with the mitogens phorbol myristate acetate or concanavalin A caused an increase in K channel density over a time course of about one day (Deutsch, Krause & Lee, 1986; DeCoursey et al.,



**Fig. 6.** (A)  $V_m$ , (B)  $C_m$ , (C)  $G_K$ , and (D)  $G_L$  with time after adherence for round (group 1) and spread (group 3) cells. Mean values were compared at 0-2 and >18 hr for each group. \*Indicates values that are significantly different from values from the same group at 0-2 hr. †Indicates values that are significantly different from values of group 1 at the same time period

1987). The increase in channel density occurred over the same time course as the increase in protein synthesis induced by mitogen.

The observed increase in membrane capacitance after plating provides the first evidence that J774.1 cells actively increase their membrane area following adherence. We found that very spread cells showed the largest increase in membrane area following adherence (>twofold), while small round



cells showed very little change. This does not imply that the membrane of smaller cells is unchanging, however. It has been shown that mouse peritoneal macrophages can pinocytose their plasma membrane at high rates (3.1%/min; Steinman, Brodie & Cohn, 1976). Membrane area is undoubtedly regulated by a balance between insertion and retrieval processes, either of which could be altered following adherence. It should also be noted that small and large cells do not necessarily represent two different subpopulations of cells. The J774 cell line is continuously dividing, and morphology may vary with cell cycle. For example, it is generally known that cells 'round up' during mitosis. The variability of membrane properties with cell cycle was not addressed here, although it is an interesting question that bears further study.

Membrane potential also increased with time after adherence, from  $-42$  to  $-58$  mV. Our results are consistent with those of Sung et al. (1985) who used  $^3\text{H}$ -tetraphenylphosphonium to measure the membrane potential of J774 cells and found that it increased from about  $-35$  to  $-70$  mV between 1 and 8 hr after plating. It is unclear whether there is a significant increase in membrane potential between 0 and 1 hr after plating. Membrane potential values of suspended J774 cells, acquired using indirect probes, have been reported to be  $-15$  mV (Sung et al., 1985),  $-25$  mV (J774.2 cells; Ehrenberg et al., 1988), and  $-36$  mV (J774.2 cells; McCaig & Berlin, 1983), and represent the average  $V_m$  of a large population of cells. The latter value is not very different from the average  $V_m$  determined in this paper for adherent cells ( $-42$  mV) shortly after plating. Given the variability in the  $V_m$  values reported for suspended cells, and the unavoidable delays in measuring  $V_m$  after adherence, this study could not resolve whether or not there is a significant hyperpolarization of J774 cells immediately after plating. However, it does demonstrate that membrane potential was positively correlated with  $G_K$ , that is, cells with larger  $G_K$  were more negative.  $G_K$  increased significantly with time after adherence, while  $G_L$  did not. We have no evidence that any other conductance was modulated following adherence. Thus, it is likely that the increase in  $G_K$  accounts for the observed increase in  $V_m$ . We cannot exclude the possibility that part of the hyperpolarization following adherence is due to an increase in the activity of an electrogenic  $\text{Na}^+/\text{K}^+$  pump, which is known to contribute  $-7$  and  $-11$  mV to the resting membrane potential of mouse spleen macrophages (Gallin & Livengood, 1983) and human monocytes (Ince et al., 1987), respectively. Increased pump activity might also lead to changes in  $[\text{K}]_i$  and thus  $E_K$ . Our value of  $E_K$  is derived from measurements of  $[\text{K}]_i$  in

suspended J774 cells (Melmed, Karanian & Berlin, 1981; Sung et al., 1985), and is approximately  $-90$  mV. However, even if resting  $V_m$  was determined solely by  $E_K$ ,  $[\text{K}]_i$  would have to double to shift  $E_K$  sufficiently to account for the observed increase in  $V_m$ .

It is important to note that, although  $G_K$  and  $V_m$  are positively correlated in J774 cells under a wide variety of conditions, the fact that resting  $V_m$  is considerably more positive than our assumed value for  $E_K$  indicates the presence of a depolarizing conductance, such as sodium ( $E_{\text{Na}} = +70$  mV, Sung et al., 1985) or chloride ( $E_{\text{Cl}} = -23$  mV, Melmed et al., 1981). In our previous studies of J774 cells (Gallin & Sheehy, 1985; Gallin & McKinney, 1988), where more negative resting  $V_m$  values were reported ( $-70$  to  $-80$  mV), the contribution of other conductances to resting  $V_m$  was probably underestimated. This was because we tended to record from larger, more spread cells, and to assume that more positive resting  $V_m$  values were due to cell damage. Although we do not know what the ionic composition of the leak conductance is at this time, further dissection of this current may explain why the resting  $V_m$  is more positive than  $E_K$ .

Protein synthesis inhibitors have been shown to block the adherence-induced expression of the surface antigen FMC17 (recently classified as CD14; Triglia et al., 1985) and the transient increase in the surface expression of Ia-antigen in human monocytes that occurs during the first 12 hr in culture (Smith & Ault, 1981). In our studies, the protein synthesis inhibitor cycloheximide did not block the initial (0 to 2 hr) expression of  $G_K$  in adherent cells, but did inhibit the subsequent increase in channel density that occurred 2 to 8 hr after adherence. Increases in  $V_m$  and  $C_m$  were also inhibited. Because we did not record from cells which had been plated in cycloheximide for  $>8$  hr, it was not possible to evaluate whether or not the increase in  $G_K$  which had occurred by one day after adherence requires further protein synthesis. In addition, because the time course of expression of  $G_K$  in J774.1 cells was similar to the time course of expression of the FMC17 antigen in human monocytes, we tested the effect of an antibody to the FMC17 antigen on  $G_K$ . However, no effects on whole-cell or single-channel  $\text{K}_i$  currents were observed.

Since 'primed' or 'activated' macrophages often exhibit increased spreading on surfaces (Pabst & Johnson, 1980) and since the degree of spreading in adherent J774.1 cells varied tremendously, it was also relevant to determine whether the membrane properties of very spread cells were different from those of nonspread cells. Spread cells had more negative resting membrane potentials than round

cells, both initially and one day after plating. Differences were also noted for the two groups of cells in their patterns of change after adherence. Only rounded cells increased  $G_K$  after adherence, and only spread cells increased capacitance. Thus, very spread cells inserted additional membrane area with time after adherence, but the increase in membrane area was not associated with a large increase in  $K_i$  channel density. The fact that  $G_K$  and capacitance varied independently of one another indicates that the increase in membrane area and expression of inwardly rectifying K channels were not necessarily linked. Final  $K_i$  channel density is determined by a balance between channel insertion or activation and channel internalization or inactivation. Our data do not differentiate between any of these processes.

The finding that spread cells did not show an increased density of  $K_i$  channels after adherence was consistent with the observation that exposing cells to LPS (which also increases cell spreading) did not increase the density of  $K_i$  channels. In fact, LPS-treated cells had significantly lower  $G_K$  values than control cells plated for comparable times. This is due in part to the increased capacitance of LPS-treated cells compared to controls, a result of the fact that LPS-treated cells are still growing, even though cell division has ceased.

Although the density of  $K_i$  channels in LPS-treated cells was lower than in control cells, resting  $V_m$  values were comparable. Clearly, there must be other differences between the two groups of cells to account for this result. Possible explanations are that LPS-treated cells have (i) a higher K permeability ratio (ii) a more negative  $E_K$  and/or (iii) increased electrogenic pump activity. However, like control cells, there was still a clear correlation between  $G_K$  and  $V_m$  in LPS-treated cells. Also, LPS-treated cells exhibited the same increase in  $K_i$  channel density, resting  $V_m$ , and capacitance following adherence as control cells did. Finally, in human monocytes, LPS has been reported to increase the percentage of cells expressing the transient outward K current (Jow & Nelson, 1989). In our studies, the  $K_o$  current was present in about 5% of the cells, but its expression was not increased by LPS.

In summary, the membrane properties of J774.1 cells change significantly after adherence, after exposure to LPS, and with cell morphology. Adherence is specifically correlated with increased  $K_i$  channel density, but only for rounded and not for spread cells. Spread cells rapidly increased their membrane area after adherence, but did not concomitantly increase  $K_i$  channel density. J774.1 cells treated for 24 hr with LPS were similar to spread cells in that they had increased capacitance but not increased  $K_i$  channel density compared to controls.

$G_K$  was positively correlated with  $V_m$  in both control and LPS-treated cells. Thus, the increase in  $G_K$  after adherence can account for the hyperpolarization which J774.1 cells undergo after plating, and an increase in surface membrane area is not necessarily linked to an increase in  $K_i$  channel density.

We thank Spencer Green for carrying out functional assays, Mr. William E. Jackson for advice on statistical analyses, and Ms. Jeanine Faw for maintaining cell cultures. Drs. Margaret Colden-Stanfield, David Livengood, and Joel Lowy critically reviewed the manuscript. Dr. Heddy Zola, Flinders Medical Center, Bedford Park, S. Australia, generously provided a sample of antibody to FMC17.

This work was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under work unit 00020. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

## References

- Berton, G., Gordon, S. 1983. Superoxide release by peritoneal and bone marrow-derived mouse macrophages. Modulation by adherence and cell activation. *Immunology* **49**:693-704.
- Bodel, P.T., Nichols, B.A., Bainton, D.F. 1977. Appearance of peroxidase reactivity within the rough endoplasmic reticulum of blood monocytes after surface adherence. *J. Exp. Med.* **145**:264-274.
- Cohn, Z.A. 1978. The activation of mononuclear phagocytes. Fact, fancy, and future. *J. Immunol.* **121**:813-816.
- DeCoursey, T.E., Chandy, K.G., Gupta, S., Cahalan, M.D. 1987. Mitogen induction of ion channels in murine T lymphocytes. *J. Gen. Physiol.* **89**:405-420.
- Deutsch, C., Krause, D., Lee, S.C. 1986. Voltage-gated potassium conductance in human T lymphocytes stimulated with phorbol ester. *J. Physiol. (London)* **372**:405-423.
- Ehrenberg, G., Montana, V., Wei, M.D., Wuskell, J.P., Leow, L.M. 1988. Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. *Biophys. J.* **53**:785-794.
- Fuhlbrigge, R.C., Chaplin, D.D., Kiely, J.M., Unanue, E.R. 1987. Regulation of interleukin-1 gene expression by adherence and lipopolysaccharide. *J. Immunol.* **138**:3799-3802.
- Gallin, E.K. 1989. Evidence for a Ca-activated inwardly rectifying K channel in human macrophages. *Am. J. Physiol.* **257**:C77-C85.
- Gallin, E.K., Livengood, D.R. 1983. Demonstration of an electrogenic Na<sup>+</sup> K<sup>+</sup> pump in mouse spleen macrophages. *Am. J. Physiol.* **241**:C184-C188.
- Gallin, E.K., McKinney, L.C. 1988. Patch-clamp studies in human macrophages: Single-channel and whole-cell characterization of two K<sup>+</sup> conductances. *J. Membrane Biol.* **103**:55-66.
- Gallin, E.K., McKinney, L.C. 1989. Ion transport in phagocytes. In: Neutrophil Physiology, M.B. Hallett, editor. CRC Press, Boca Raton (FL).
- Gallin, E.K., Sheehy, P.A. 1985. Differential expression of inward and outward potassium currents in the macrophage-like cell line J774.1. *J. Physiol. (London)* **369**:475-499.

- Gordon, S., Unkeless, J.C., Cohn, Z.A. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. *J. Exp. Med.* **140**:995-1010
- Haskill, S., Johnson, C., Eierman, D., Becker, S., Warren, K. 1988. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J. Immunol.* **140**:1690-1694
- Ince, C., Thio, B., van Duijn, B., van Dissel, J.T., Ypey, D.L., Leijh, P.C.L. 1987. Intracellular  $K^+$ ,  $Na^+$ , and  $Cl^-$  concentrations and membrane potential in human monocytes. *Biochim. Biophys. Acta* **905**:195-204
- Jow, B., Nelson, D.J. 1989. Outwardly rectifying  $K^+$  current as a marker of cellular activation in human macrophages. *Biophys. J.* **55**:539a
- Karnovsky, M.L., Lazdins, J.K. 1978. Biochemical criteria for activated macrophages. *J. Immunol.* **121**:809-813
- Kunkel, S.L., Duque, R.E. 1983. The macrophage adherence phenomenon: Its relationship to prostaglandin  $E_2$  and superoxide anion production and changes in transmembrane potential. *Prostaglandins* **26**:893-901
- Kurland, J.L., Bockman, R. 1978. Prostaglandin  $E$  production by human blood monocytes and mouse peritoneal macrophages. *J. Exp. Med.* **147**:952-957
- McCaig, D.J., Berlin, R.B. 1983. Transmembrane potential of J774.2 mouse macrophage cells measured by microelectrode and ion distribution methods. *Experientia* **39**:906-907
- McKinney, L.C., Gallin, E.K. 1988. Inwardly rectifying whole-cell and single-channel  $K$  currents in the murine macrophage cell line J774.1. *J. Membrane Biol.* **103**:41-53
- Melmed, R.N., Karanian, P.J., Berlin, R.D. 1981. Control of cell volume in the J774 macrophage by microtubule disassembly and cyclic AMP. *J. Cell. Biol.* **90**:761-768
- Midoux, P., Petit, C., Pellen, P., Toujas, L., Monsigny, M., Roche, A.-C. 1989. Macrophage antigens associated with adhesion: Identification by a monoclonal antibody specific for Lewis lung carcinoma cells. *Exp. Cell Res.* **183**:168-178
- Morland, B., Kaplan, G. 1977. Macrophage activation in vivo and in vitro. *Exp. Cell Res.* **108**:279-288
- Okada, M., Kishimoto, T., Igarashi, T., Teranishi, T., Yamamura, Y. 1978. LPS-or 8Br-cyclic AMP-induced production of T cell-activating factor(s) in macrophage tumor cell line J774.1. *J. Immunol.* **120**:1097-1101
- Pabst, M.J., Hedegaard, H.B., Johnston, R.B., Jr. 1982. Cultured human monocytes require exposure to bacterial products to maintain an optimal oxygen radical response. *J. Immunol.* **128**:123-128
- Pabst, M.J., Johnston, R.B. 1980. Increased production of superoxide anion by macrophages exposed to in vitro muramyl dipeptide or lipopolysaccharide. *J. Exp. Med.* **151**:101-114
- Pofit, J.F., Strauss, P.R. 1977. Membrane transport by macrophages in suspension and adherent to glass. *J. Cell. Physiol.* **92**:249-256
- Ralph, P., Nakoinz, I. 1977. Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histiocytic or macrophage tumor cells in culture. *Cancer Res.* **37**:546-550
- Randriamampita, C., Trautmann, A. 1987. Ionic channels in murine macrophages. *J. Cell Biol.* **105**:761-769
- Smith, B.R., Ault, K.A. 1981. Increase of surface Ia-like antigen expression on human monocytes independent of antigenic expression. *J. Immunol.* **127**:2020-2027
- Steinman, R.M., Brodie, S.E., Cohn, Z.A. 1976. Membrane flow during pinocytosis. A stereologic analysis. *J. Cell Biol.* **68**:665-687
- Sung, S.S., Young, J.D.E., Origlio, A.M., Heiple, J.M., Kabback, H.R., Silverstein, S.C. 1985. Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic  $[Ca^{2+}]$ , and inhibits phagocytosis in mouse macrophages. *J. Biol. Chem.* **260**:13442-13449
- Triglia, T., Burns, G.F., Werkmeister, J.A. 1985. Rapid changes in surface antigen expression by blood monocytes cultured in suspension of adherent to plastic. *Blood* **65**:921-928
- Ypey, D.L., Clapham, D.E. 1984. Development of a delayed outward-rectifying  $K^+$  conductance in cultured mouse peritoneal macrophages. *Proc. Natl. Acad. Sci. USA* **81**:3083-3087
- Zabrenetzky, V., Gallin, E.K. 1988. Inositol 1,4,5-trisphosphate concentrations increase after adherence in the macrophage-like cell line J774.1. *Biochem. J.* **255**:1037-1043
- Zacharchuk, C.M., Drysdale, B.-E., Mayer, M.M., Shin, H.S. 1983. Macrophage-mediated cytotoxicity: Role of a soluble macrophage cytotoxic factor similar to lymphotoxin and tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* **80**:6341-6345

Received 28 August 1989; revised 2 January 1990